Changes in Leaf Internal Structure of Rice Plants to Application of Varied Rates of Nitrogen Fertilizer

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ABSTRACT

Experiments were conducted to examine changes in leaf internal structure of field-grown rice plants (*Oryza sativa* L. cv. TNG 67) to varied rates of nitrogen (N) fertilizer, from 0 to 180 kg N ha^{-1} with 60 kg N ha^{-1} intervals, applied to the experimental plots during the first and the second cropping seasons of 2001. Results showed that aboveground N content of rice plants measured in the panicle initiation stage was related to the increase of N rates applied to the paddy and a linear fashion was displayed in the range of N application rates. A diversity of leaf anatomical characteristics was observed in leaves from tagged plants of varied N status grown in both crops, especially between plants treated with 0 and 180 kg N ha^{-1}. The packing and arrangement of starch granules within parenchyma cells changed in leaves treated with varied N rates. Starch granules were loosely distributed and packed in plants of higher N content (180 kg N ha^{-1}) relative to those of lower N one (0 kg N ha^{-1}). Leaf thickness increased progressively with increasing aboveground N content and leaf water content (LWC) changed in a curvilinear trend. Under normal growth conditions leaves may roll to a certain extent owing to water loss through transpiration even with sufficient water supply, while this phenomenon was relieved in plants applied with heavy rate of N fertilizer. A higher value of leaf rolling index (LRI) was computed in high N-treated plants, showing in a linear trend. Changes in the ratio of bulliform/mesophyll to aboveground N content were a quadratic function. Results imply that rice plants have the ability and plasticity to regulate their leaf internal structure and thus may enable them adapting to soils varying in N supply in both cropping seasons.

Key words: Leaf internal structure, Leaf anatomical characteristics, Rice, Nitrogen application rate.

不等量氮肥施用導致水稻葉片內部構造之改變

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摘要

本項研究利用生育期間施予不等量氮肥方式(於0-180 kg N ha^{-1}範圍每隔60 kg N ha^{-1} 劃分四不同等級)，探討不同含氮狀態之田間生長稻株(*Oryza sativa* L. cv. TNG 67)葉片內部構造之變化。根據試驗結果，穗形成啟始期之稻株地上部含氮狀態與氮肥施用量之間呈現顯著直線正相關，無論2001年一、二期稻作皆出現類似關係。不同含氮狀態之稻株顯示出不等葉片解剖特徵差異，尤以0 kg N ha^{-1}及180 kg N ha^{-1}兩處理差異最為明

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顯。諸如葉肉細胞內澱粉顆粒排列及分佈，即隨著氮肥施用量而異。相較於含氮狀態較低之葉肉細胞(如0 kg N ha^{-1}處理)，高含氮狀態之葉肉細胞(如180 kg N ha^{-1}處理)其澱粉顆粒排列及分佈較為鬆散。葉片厚度伴隨稻株地上部含氮狀態之升高而增加，葉片水分含量則呈現二次曲線改變。儘管水分不虞匱乏，常態環境下葉片因蒸散之水分損耗可能出現不等捲曲現象，惟施用較高氮肥稻株之葉片其捲曲情形較為緩和。進一步計算葉片捲曲指數(leaf rolling index, LRI)，發現含氮較高者之LRI值較大(即葉片捲曲程度較小)，為一線性關係，而泡狀細胞面積與葉肉層面積比值(bulliform/mesophyll ratio)與地上部氮素含量之間則呈現二次曲線關係。綜合本試驗之研究結果，顯示稻株具有調節葉片內部構造之能力與可塑性，使其能夠自我調適存活於不等土壤氮素供應環境。

關鍵詞： 葉片內部構造、葉片解剖特徵、水稻、氮肥施用量。

INTRODUCTION

Rice is becoming a model plant in monocotyledons and cereal crops (Itoh *et al*. 2005). For better understanding of the rice plant, it is necessary to further clarify the developmental progress, both morphologically and anatomically, and the underlying mechanisms to survive and reproduce across a range of environments. The most notably stresses encountered by rain-fed and irrigated lowland rice in Taiwan are water deficiency and nutrient limitation. With the intermittence of anaerobic and aerobic conditions in the field, nutrient uptake efficiency will be interrupted periodically due to adverse effects of soil oxidation/reduction cycles (Huguenin-Elie *et al*. 2003, Seng *et al*. 2004), and thus changes in plant morphological and anatomical features and physiological functions occur.

Botanically, a leaf, usually green, flat and thin allowing light to penetrate through, is an aboveground plant organ for photosynthesis, by which light energy is captured in the form of sugar molecules. Leaf is also the principal sites where respiration, transpiration, and guttation take place in most plants. Rice leaves arise from the node and are borne at an angle in an alternate phyllotaxy (Yoshida 1981). Varieties differ in leaf number, but a typical leaf consists of leaf sheath, auricle, ligule, and leaf blade. The leaf blade, or called leaf lamina, is the expanded portion of the leaf continuous with the leaf sheath which encloses the culm above the node in varying length, form and tightness. There is the tremendous variety shown in leaf blade structure from species to species, yet the basic organization of internal tissues and the developmental pathways are similar. The epidermis, mesophyll, and veins are three basic tissues of a leaf blade of most vascular plants including rice.

The epidermal cells are arranged regularly in rows forming the boundary of leaf blade from the external world, and can protect against water loss, regulate gas exchange, secret metabolic compounds, and retain water in the leaf (Yoshida 1981). The upper (adaxial) and lower (abaxial) surfaces are usually coated with a waxy cuticle and may have different construction and functions. The mesophyll, or called a parenchyma tissue, is located in-between the upper and lower layers of epidermis. This assimilation tissue is where most of the chloroplasts are found and is the primary location of photosynthesis. The vascular tissues within leaf veins include xylem, which transports water and inorganic compounds into the leaf, and phloem, which transports organic compounds produced by photosynthesis away from the leaf.

Nitrogen (N) is the most essential element affecting crop productivity among plant nutrients. Application of N fertilizer is a necessary measure in enhancing and stabilizing crop growth and yield production (Martens 2001). To rice crop, yield is known to closely related to N status prior to heading (Cui and Lee 2002, Ntanos and Koutroubas, 2002), and N topdressing at panicle initiation/formation stage is of most crucial for its yield production (Nguyen and Lee 2006). In this regard, assessing plant N status remotely, by remote sensing techniques, or structurally, with microscopic methods, at this critical period can provide the needed information for a variable rate fertilizer application and for explaining why and how plant response to N application. If there is an

appropriate indicator link together rice growth and N condition, growers are able to make suitable decision on a reliable trait.

This study attempted to categorize the internal structural changes of rice leaf blade in the panicle initiation stage upon application of varying rates of N fertilizer based exclusively on the anatomical viewpoints. The importance of the differential plasticity of leaf anatomical characteristics in response to variation in plant N status was also discussed.

MATERIALS AND METHODS

A semi-dwarf japonica type cultivar Tainung 67 (*Oryza sativa* L.) known to susceptible to N application rates was chosen for this study. With the use of recommendation rate of N fertilizer, grain yield of this cultivar can easily reach more than 6 ton ha^{-1}. Field experiment was carried out in the experimental farm of Taiwan Agricultural Research Institute at Wufeng, Taichung Hsien, during the first and the second cropping seasons of 2001. Seedling were machine-transplanted, 3-5 plants per hill, to 0.3 m wide north-south rows with plant distance of 0.18 m and resulted in a population density of about 185,000 hills ha^{-1}. The transplanting day was on 6 March 2001 for first crop and on 12 August 2001 for second crop, respectively.

Three field plots (replicates), 0.5 ha for each, were located on a loam soil of less than 1.50 m deep and a pH of 4.53 to 5.08. Each plot was divided into four equal-area blocks (25 m wide and 50 m long) laid out side-by-side. The soil total N was less than 0.0008 kg kg^{-1} and the organic matter (OM) was in a range of 0.011-0.019 kg kg^{-1}. Four different rates of N fertilizer, in the form of ammonium sulfate (21% N, Taiwan Fertilizer Corp., Taiwan), were assigned individually to these four blocks. From Block 1 to Block 4, N fertilizer was applied at a rate in the sequence of 0, 60 120, and 180 kg N ha^{-1}, respectively. Each quantity was split into four doses: 25% of the amount distributed as preplanting basal 5 days before transplanting, 20% as the first top dressing applied 1 week (second) or 2 weeks (first crop) after transplanting (WAT), 30% as the second top dressing added 3 WAT (second crop) or 4 WAT (first crop), and the rest 25% was used 20 days before heading (ca. 6-8 WAT). For all N treatments, P_2O_5 in a rate of 70 kg ha^{-1} was incorporated into soil before transplanting. Dipotassium oxygen (K_2O), in a rate of 70 kg ha^{-1}, was distributed 40% for preplanting basal, 20% for first top dressing, 20% for second top dressing, and 20% for preheading dressing, respectively.

Herbicide butachlor (5% granule, 2-Chloro-2',6'-diethyl-N-(butoxymethyl) acetate) was applied in a rate of 1.5 kg a.i. ha^{-1} within 1 WAT and bentazon (44.1% solution, 200X, sodium salt of 3-isopropyl-1H-2,1,3-benzothiadiazin-4-(3H)-one-2,2- dioxide) in a rate of 1.3 L a.i. ha^{-1} was applied 3 WAT. Pesticide cartap (6% granule, 1.8 kg a.i. ha^{-1}, 1,3-bis-(carbamoylthio)-2-(N,N-dimethylamino)-propane hydrochloride) was used to control stem borer and carbofuran (40% WP, 800 X, 0.48 L a.i. ha^{-1}, 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) was used to control planthopper [*Nilaparvata lugens* (Stål)], leaffolder [*Cnaphalocrocis medinalis* (Guenee)] and others. Fungicide validamycin A (50% S, 1000X, 0.5 L a.i. ha^{-1}) was used to protect from infection by sheath blight (caused by *Rhizoctonia solani* Kühn). All were sprayed during rice growth as needed.

Tagged plants from targeted regions of different N-treated blocks were sampled in the stage of panicle initiation on 20 April 2001 for first crop and 24 September 2001 for second crop. The weather on these two days was sunny with a light wind less than 2 m s^{-1}. Six hills of rice were sacrificed on each sampling and fresh weights of plant parts of the aboveground (leaf blades and culms) were obtained, and dry weights were measured after oven-dried at 80℃ for 72 h before milling. The powdered samples were passed through 0.1 mm mesh for determination of total N content of the aboveground plant parts, by using the modified Kjeldahl method reported by Yang *et al*. (2003a, 2003b).

Another three hills of each treatments were sampled at the same targeted regions in the next morning to minimize dehydration of the dissected tissue and the diurnal swelling/shrinkage cycle (McBurney 1992), and the leaf anatomical characteristics were determined. Six segments of 1-cm long were cut consecutively 10-cm away from the apex of upper-most fully expanded leaf blades on the main culms and were fixed in FAA

solution (formaldehyde:acetic acid:70% ethanol = 5:5:90, v/v/v) immediately (Sass 1958), and then dehydrated in a TBA (tertiary butyl alcohol) series. The completely dehydrated cross-sectional segments were immersed into paraffin in 60-62℃ oven. The process of paraffin infiltration was made 3 to 4 times before embedding of samples with a paraffin dispenser (Lecia EG 1120, Leica Microsystems Nussloch GmbH, Germany). Cross-sections of 10-μm thick prepared from a rotary microtome (Leica RM 2135) were stained with 1% safranin-fast green, for discriminating leaf internal structure, and PAS (periodic acid-Schiff's reaction for carbohydrates) (Jensen 1962), for differentiating starch granules, and the slides were mounted in balsamo resin (ASSISTENT-Histokitt, Hecht Assistant Company, Germany).

The photographs were taken under a light microscope (model BH-2, Olympus Corp., Japan) operated at magnifications of 150, 300, and 600 X using a digital camera (E5400, Nikon Corp., Japan). Micrographs were analyzed with the image analysis software SimplePCI (Compix Inc., Imaging Systems, USA) and the desired anatomical characteristics of leaf (Fig. 2), including leaf width, leaf thickness, bulliform cells and mesophyll layer, were identified and quantified in order to correlate against aboveground N content. Three groups of sections from each treatment were selected and the values of anatomical measurements of each group were averaged. Leaf thickness was measured from the traversed distance across the mesophyll of leaf blade, and values from more than twelve locations along mesophyll layer of each segment were averaged and the mean of three replicates was used as representative. Leaf rolling index (LRI) was determined by dividing the distance of cross axis perpendicular to the axis of midrib of rolling leaves to the width of leaf section, where $0 < LRI \leqq 1$ (1= flat leaf), and was used as an indicator of the magnitude of leaf rolling after dissection. Size ratio of bulliform cells to mesophyll was computed for plants treated with different N rates, and was used as a symbol of leaf movement strength (Buléon *et al*. 1998); large ratio represent stronger leaf movement strength. Leaf water content (LWC) was calculated as LWC (%) = [(fresh weight of leaf blade-dry weight of leaf blade)/dry weight of leaf blade] × 100%, by using leaf blades excised from the sampled plants.

The statistics were performed where applicable to analyze differences between N treatments, and the yielded results and the graphs were plotted by using SigmaPlot 8.0.2 (SPSS ASC BV, The Netherlands). All variables were determined and averaged in each replicate and the mean and standard error of three replicates were calculated if necessary.

RESULTS AND DISCUSSION

To produce rice plants with varied N status, different rates of N fertilizer, from 0 to 180 kg N ha^{-1} with 60 kg N ha^{-1} intervals, were applied to experimental plots during the growing seasons. Rice plants applied with a higher rate of N fertilizer can be expected to attain a higher level of N status. Nitrogen content of aboveground plant parts, in the panicle initiation stage (data not shown), increased linearly with the increase of N application rates in both first (r=0.936*) and second (r=0.997**) crops of 2001. Results were similar to that reported by Chang *et al.* (2004), the aboveground N content of rice plants measured in the panicle initiation stage was related to the increase of N rates applied to the paddy and a linear fashion was displayed in the range of N application rates. The increase of plant N status appears one of the primary responses to the application of N fertilizer.

The micrographs (×600) illustrated in Fig. 1 shows the typical anatomy of a rice leaf blade from plants of the panicle initiation stage under local recommended N application rate (120 kg ha^{-1}). There are three basic tissues to see in the leaf internal structure, i.e., epidermis, mesophyll, and vascular bundle. As observed in the cross-sections, the upper (adaxial) and lower (abaxial) epidermal cells form the boundary of leaf blade, as a continuous covering over the leaf. The mesophyll is located in-between the epidermal layers mostly made up of fold parenchyma cells, which are densely packed with chloroplasts giving the dark green appearance when stained with 1% safranin-fast green. The vascular bundle within leaf veins includes xylem and phloem, and is arranged parallel to each other throughout the leaf. Moreover, there are many sets of bulliform

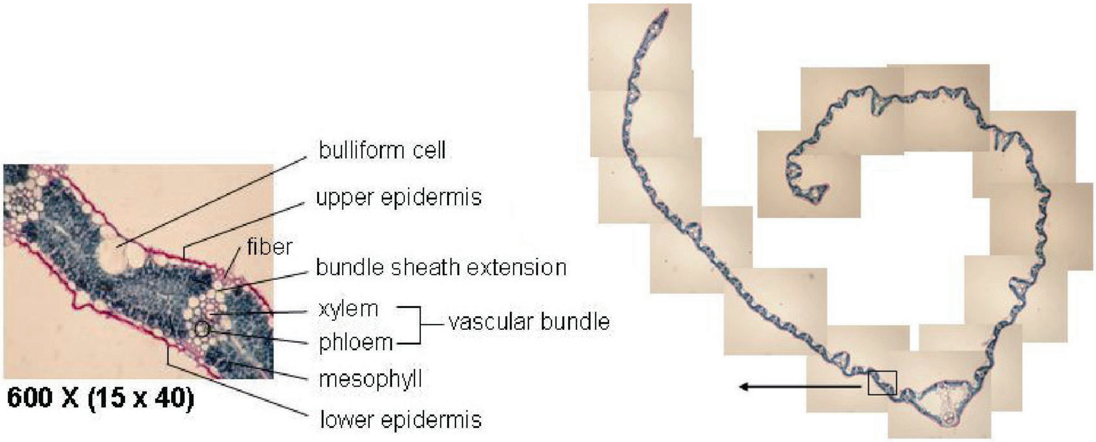

Fig. 1. The anatomical characteristics of leaf blade observed in cross-sections (×600) from rice plants (*Oryza sativa* L. cv. TNG 67) grown at the panicle initiation stage under local recommended N application rate 120 kg ha^{-1}.

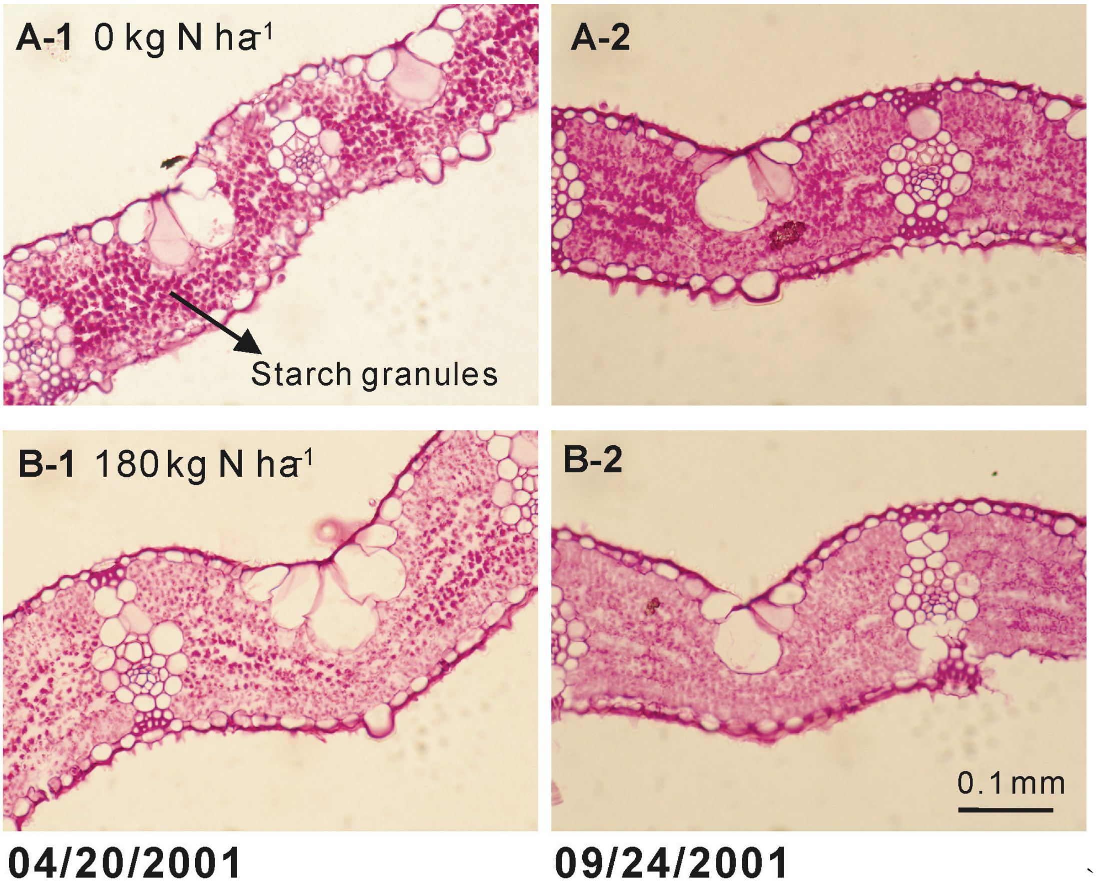

Fig. 2. Differences in starch granules packing and distribution in segments of epidermal cells observed in leaf cross-sections (×300) from rice plants (*Oryza sativa* L. cv. TNG 67) grown at the application rates of 0 and 180 kg N ha^{-1}, respectively. ×600

cells, also known as motor cells, recognized in the upper epidermis near the midrib. The bulliform cells are highly vacuolated thin-walled cells larger than the epidermal cells and have a particular mechanism to allow the leaf blade to curl or roll up rather than merely fold.

Although leaf anatomy is genetically controlled, environment also plays an important role in its expression. The alterations of plant N status by varied N application rates changed plant architecture (Lee *et al*. 2002) as well as leaf internal structure. In the case of starch granules investigated in this study, the packing and arrangement of these granules within parenchyma cells changed in leaves treated with varied N rates, especially between plants treated with 0 and 180 kg N ha^{-1} (Fig. 2). As exhibited from colorings of cross-section segments with PAS method, starch granules were shown loosely distributed and packed in the parenchyma cells in plants of higher N content (180 kg N ha^{-1}) relative to those of lower N one (0 kg N ha^{-1}). The dispersed and less condensed starch granules occurred in leaves of higher N may be considered as a piece of evidence indicating the exhaustion of stored reserves in starch granules of leaves to support a vigorous plant growth.

There were also noteworthy changes morphologically, in terms of leaf thickness, leaf width and leaf rolling, by applying different rates of N fertilizer. The mean thickness of leaf blade altered significantly in plants developed from different N status (Fig. 3). Leaf thickness increased linearly with increasing aboveground N content. In the measured range of aboveground N content, leaf LWC changed in a curvilinear trend (Fig. 4), indicating that there was a limit in water accumulation in the leaf upon the addition of N level. As both leaf thickness and water content were enhanced by the abundant N supply in coincident with the observed vigorous growth of plants, results suggest that an improved N content may in favor of water absorption to the leaf and expansion of mesophyll layer.

Under normal growth conditions leaves may roll to a certain extent owing to water loss through transpiration even with sufficient water supply. Such phenomenon was confirmed in this study and was relieved in plants of higher N status (Fig. 5). As the micrographs illustrated, lesser extent of leaf rolling was observed in leaves treated with 180 kg N ha^{-1} than with 0 kg N ha^{-1}. By further plotting values of LRI against aboveground N content, results showed a positive linear relationship in both cropping seasons (Fig. 6). Plants with higher aboveground N content had increased LRI values, i.e., with more flattened leaves. In addition, the relationship between bulliform/mesophyll ratio and aboveground N content was best fitted to a quadratic function in both crops (Fig. 7). The ratio increased 13% from

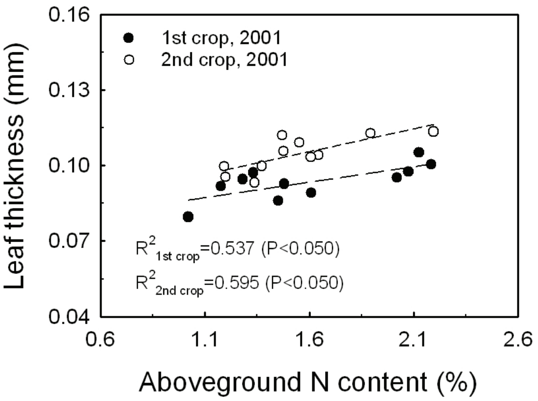

Fig. 3. Changes in the thickness of leaf blade to aboveground N content for rice plants (*Oryza sativa* L cv. Tainung 67) grown in the first and the second cropping seasons of 2001.

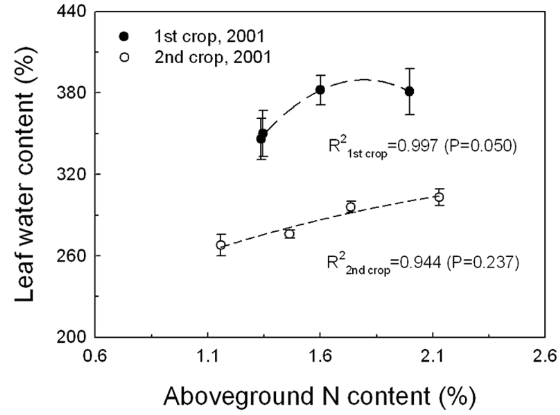

Fig. 4. Changes in leaf water content (LWC) to aboveground N content for rice plants (*Oryza sativa* L cv. Tainung 67) grown in the first and the second cropping seasons of 2001.

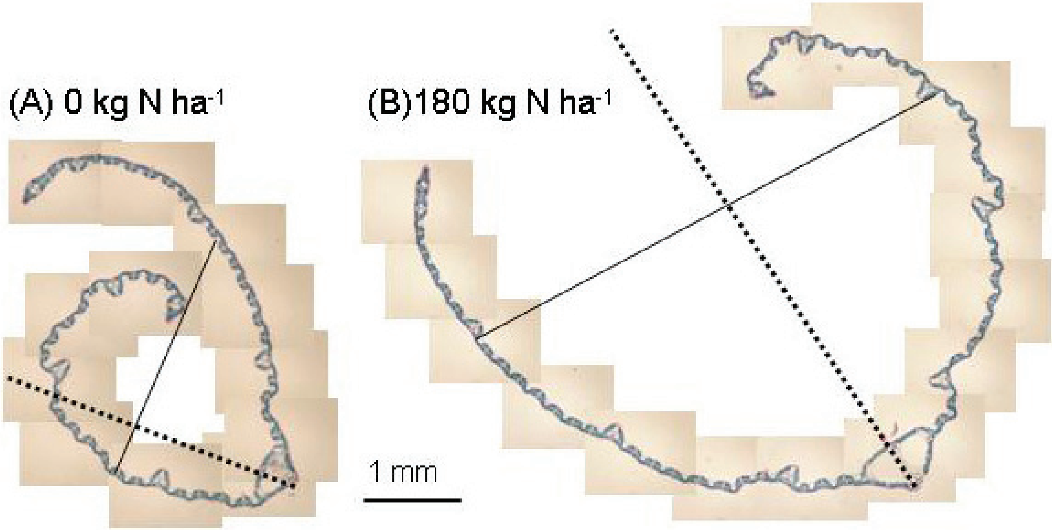

Fig. 5. Features of leaf rolling observed in leaf cross-sections from the upper-most fully expanded leaves of rice plants (*Oryza sativa* L. cv. TNG 67) grown the application rates of 0 and 180 kg N ha^{-1}, respectively. ×150

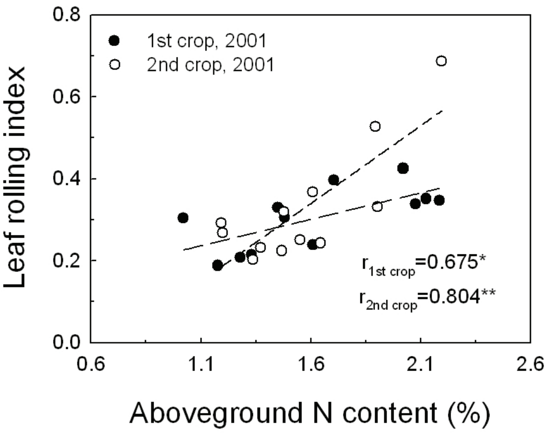

Fig. 6. Changes in leaf rolling index (LRI) to aboveground N content for rice plants (*Oryza sativa* L cv. Tainung 67) grown in the first and the second cropping seasons of 2001.

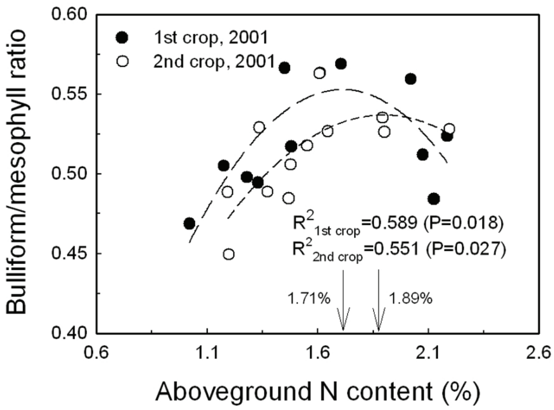

Fig. 7. Changes in bulliform/mesophyll ratio to aboveground N content for rice plants (*Oryza sativa* L cv. Tainung 67) grown in the first and the second cropping seasons of 2001.

1.18% to 1.89% of aboveground N content in leaves of second crop relative to 21% from 0.98% to 1.71% of aboveground N content in leaves of first crop, and decreased hereafter. Since plant growth is known to be promoted by the application of N and both length and width of leaf growth zone were improved by the increased N status (Lee *et al.* 2002, Mae and Ohira 1981), the positive correlations of LRI and bulliform/ mesophyll ratio to aboveground N content suggest that application of higher N rate not only provides a stronger expansion strength/vigor to stimulate plant growth but also promote a size increase of bulliform cells and an outward tension

of leaf blade. An enlargement of bulliform cells within mesophyll layer in response to a higher N is logical in that function of these bubble shaped cells is to control leaf movements (Buléon *et al*. 1998) and more turgid leaves with larger leaf area were observed in plants with higher N level. The bulliform cells must fill with water under a higher N status to impede rolling of leaves. However, the quadratic relationship between bulliform/ mesophyll ratio and aboveground N content infers that there is a limit for the enlargement of bulliform cells and the improvement of mesophyll layer can also counterbalance the increase of the ratio.

Morphological and anatomical changes in leaves, such as increased leaf number, epidermis layer and cell wall thickness, lower specific leaf area, and altered stomatal frequency, have been reported in many plant species grown under low temperature conditions (Hunner *et al*. 1981), and have been related to the increased freezing tolerance (Fowler *et al*. 1981). Leaf thickness and mean mesophyll cell size were found directly related to light-saturated photosynthesis per unit leaf area in C_3 grasses (Wilson and Cooper 1967, Charles-Edwards *et al*. 1974, Nobel *et al*. 1975). A better photosynthetic rate was also demonstrated in C_3 plants of thicker leaves and higher ratio of total surface area of the mesophyll cells to leaf area (Nobel *et al.* 1975, Hattersley 1984, Araus *et al.* 1986), similar to that found in corn, a C_4 species (Louwerse and Zeerde 1977, Moreno-Sotomayor *et al*. 2002). These results provide a clear picture that CO_2 movement through stomata to the carboxylation sites (mesophyll cells) is facilitated in conditions of large area ratios of mesophyll cells to leaf and thicker leaves, and thus enhance photosynthetic rate. Apparently similar morphological and anatomical changes occurred in rice leaves caused by differences in plant N status may be functionally relevant to physiological modifications and growth progression and account for the diversity in growth manner.

Further, as plants with higher N status prior to heading are in good position of a better yield potential (Cui and Lee 2002, Ntanos and Koutroubas 2002, Chen and Yang 2005), a higher N level during the vegetative growth phase plus an appropriate N topdressing at panicle initiation stage can provide a push power leading for a fruitful yield production (Nguyen and Lee 2006). Nevertheless, leaves curled in the lower N may have ecophysiological meaning in reducing water loss through transpiration and gas exchange rate through stomata to counterbalance the limited N supply available for growth. Maintaining a lower N level, therefore, may be advantageous for plants to pass through drought period and one way to survive in fields deficient in water supply. Such an ability and plasticity to regulate their leaf internal structure and photosynthetic rate enables rice plants adapting to soils varying in N supply in both cropping seasons.

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